EXHIBIT A

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Cells transformed by ODC, c-Ha-*ras* and v-*src* exhibit MAP kinase/Erk-independent constitutive phosphorylation of Sos, Raf and c-Jun activation domain, and reduced PDGF receptor expression

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While it is known that the constitutive activity of a variety of signal transduction molecules leads to cell transformation, a key unresolved question is whether these wirings converge to a common intermediate(s) that dictates transformation. In this study, we investigated whether NIH3T3 and Rat-1 cells transformed by human ornithine decarboxylase (ODC), c-Ha-*ras*^{Val12} and temperature-sensitive v-*src* oncogene display common alteration(s) in the components that relay PDGF-mediated signals in normal fibroblasts. The *ras*- and ODC-transformed cells did not show constitutively elevated tyrosine phosphorylation of the phospholipase Cγ-1 (PLCγ-1), RasGTPase-activating protein (GAP), phosphotyrosine phosphatase Syp, Shc proteins, and phosphatidylinositol 3-kinase (PI3-K) or activation of the MAP kinase (Erk1 and Erk2), p70 S6 kinase or the Janus protein tyrosine kinase (JAK) and signal transducer and activator of transcription (STAT) protein-1 pathways. Instead, the Ras nucleotide exchange factor Sos-1 and Raf-1 kinase exhibited constitutive phosphorylations, as deduced from their electrophoretic mobility shifts in polyacrylamide gels. Hence a kinase distinct from Erk1 and Erk2, previously known to feedback phosphorylate Sos-1 and Raf-1, is responsible for the phosphorylation of these molecules in the transformants. We also demonstrate that the *ras*- and ODC-transformed cells exhibit loss of both the PDGF α- and β-receptors, while the v-*Src*-transformants show a predominant reduction in the β-receptors. Moreover, all the transformed cell lines were found to display a constitutive increase in phosphorylation of c-Jun on serines 63 and 73, which appears to be governed by an as yet unknown kinase.

Keywords: transformation; ODC; Ras; Src; MAP kinase; c-Jun phosphorylation

Introduction

The proliferation of normal cells is tightly regulated by specific growth factors in the environment. Growth factors bind to and activate their cognate cell-surface receptors, which subsequently recruit and activate a distinct set of cytoplasmic signaling molecules that relay signals from the plasma membrane to the cell interior and nucleus. By contrast, transformed cells have escaped from the control of growth factors and can grow (at least to a certain degree) autonomously due to the aberrant intrinsic signaling elicited by activated oncogenes or inactivated tumor suppressor genes. Despite the remarkable progress made in understanding the oncogenic signaling cascades, the ultimate mechanisms behind the cellular transformation have, however, remained elusive. Supposing that a transformed cell should display constitutive or inappropriate activation of one or more components in the signaling pathway to gain independence of the upstream growth signals, we decided to search for signaling events altered in common in different transformed cells using normal cells untreated or treated with platelet-derived growth factor (PDGF) as a reference.

PDGFs are the major growth factors for fibroblasts. They are dimeric proteins consisting of A and B chains that can occur in all possible combinations *in vivo*. The PDGF B-chain shows over 90% homology to the transforming protein encoded by the v-*sis* oncogene. The normal PDGF B-chain also has oncogenic activity, while the PDGF A-chain has only a weak transforming potential. Two distinct types of receptors have been identified for the PDGFs, designated PDGF α- and β-receptor, which associate noncovalently to form homo- or heterodimers upon ligand binding. PDGF-AA binds specifically to the α-receptor and PDGF-BB binds both the α- and β-receptors with high affinity (Heldin, 1992). Dimerization of receptors triggers their intrinsic tyrosine kinase activity and results in auto/transphosphorylation of the receptors on multiple tyrosine residues. The phosphorylated tyrosine residues in the cytoplasmic portion of the receptor then serve as docking sites for a number of different effector signaling molecules containing SH2 and/or SH3 domains (mediating specific protein-protein interactions), such as phospholipase Cγ-1 (PLCγ-1), Ras GTP-ase-activating protein (GAP), the p85 subunit of phosphatidylinositol 3-kinase (PI3-K), Src family kinases, Grb-2, Nck, Shc and phosphotyrosine phosphatase-1D (Syp) (Mayer and Baltimore, 1993; Pawson and Schlessinger, 1993).

Cells transformed by *ras*, the most commonly activated oncogene in human cancers, have been found to become refractory to PDGF stimulation and display loss or suppression of the PDGF-stimulated phospholipase C activity (Alonso *et al.*, 1988; Parries *et al.*, 1987), Ca^{2+} mobilization (Benjamin *et al.*, 1988) and expression of growth regulatory genes, like c-*myc*, c-*fos* and ornithine decarboxylase (ODC) (Hölttä *et al.*, 1988; Lin *et al.*, 1988; Zullo and Faller, 1988). The *ras*-transformed cells have, however, been found to display relatively

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normal levels of PDGF receptors (Parries *et al.*, 1987; Rake *et al.*, 1991; Zullo and Faller, 1988). According to previous findings the attenuation of PDGF-mediated signal transduction by *ras* results from suppressed receptor autophosphorylation (Rake *et al.*, 1991; Tomáška and Resnick, 1993), which would be expected to interfere with the binding of the signaling molecules to the receptor. Indeed, it has been reported that in *ras*-transformed cells RasGAP does not become associated with the PDGF receptor or increase its phosphotyrosine content in response to PDGF stimulation (Kaplan *et al.*, 1990). However, in another study (Molloy *et al.*, 1992) PDGF stimulation of *ras*-transformed cells was found to result in increased tyrosine phosphorylation of RasGAP similar to that found in normal cells. In addition, PLC*γ*-1 has been reported to associate with the PDGF receptor and become phosphorylated almost normally in *ras*-transformed cells (Kaplan *et al.*, 1990), in spite of the documented loss or reduction of the PLC activity. Further, there are conflicting results as to whether the mitogenic response to PDGF is altered (Molloy *et al.*, 1992; Parries *et al.*, 1987) or not (Benjamin *et al.*, 1988; Lin *et al.*, 1988) following *ras* transformation. Controversial results also exist regarding the specificity of these changes to *ras* transformation. In v-*src*-transformed cells PDGF has been documented to stimulate normally phosphoinositide hydrolysis (Parries *et al.*, 1987) and c-*fos* expression (Lin *et al.*, 1988), but a complete lack of the PDGF-stimulated inositol phospholipid hydrolysis has been reported as well (Alonso *et al.*, 1988).

In order to resolve these conflicting data and sort out the signaling perturbations ultimately dictating cell transformation, we studied the activation state of different signaling molecules in mouse and rat fibroblasts transformed by c-Ha-*ras*^{Val12}, human ODC and v-*src* oncogene relative to the PDGF-stimulated normal fibroblasts. It is notable here that ODC may be a central player in the signaling of many oncoproteins, as its activity is constitutively elevated in cells transformed by different chemical carcinogens (Pegg, 1988) and oncogenes such as v-*src*, *neu* and *ras* (Gazdar *et al.*, 1976; Hölttä *et al.*, 1988; Sistonen *et al.*, 1989a, b). Further, the products of the central c-*myc* and c-*fos* oncogenes transactivate ODC (Bello-Fernandez *et al.*, 1993; Wrighton and Busslinger, 1993) and the v-*src*-induced cell transformation can be blocked by transcription of ODC antisense RNA or with a specific inhibitor of ODC (Auvinen *et al.*, 1992; Hölttä *et al.*, 1993). The ODC-induced signaling events have remained almost fully uncharacterised. The present data show that the *ras*-, v-*src*- and ODC-transformed cells display an attenuated response to PDGF stimulation with regard to tyrosine phosphorylation and activation of most of the signal transduction molecules, and that this is not due to their being constitutively activated. However, all the transformants displayed a constitutive phosphorylation (electrophoretic mobility shift) of Raf-1 and Sos-1, which is caused by a protein kinase(s) distinct from Erk1 and Erk2. Most importantly, we also found a constitutive phosphorylation of c-Jun on its aminoterminal activation domain in all these transformed cells, which is not due to JNK activation solely. In addition, we found that the *ras*-transformed cells show a repression of the PDGF receptor expression, particularly of the type *β*-receptor correlating with the degree of transformation. The ODC-transformed cells likewise exhibit a marked reduction in both receptors, while the v-*src*-transformed cells show a predominant suppression of PDGF *β*-receptors.

Results

Stimulation of protein tyrosine phosphorylation in normal NIH3T3 cells by PDGF-AA and -BB

Quiescent NIH3T3 fibroblasts (starved in 0.5% serum for 24 h) were treated initially with different concentrations (0–100 ng/ml) of the PDGF-AA and PDGF-BB isoforms for 15 min, and the tyrosyl phosphorylation of proteins was analysed by SDS-polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotting with anti-phosphotyrosine antibodies. Increasing the concentration of both PDGF-AA and PDGF-BB up to 30 ng/ml resulted in a progressive increase in the tyrosine phosphorylation of most of the proteins, following which relatively little, if any, further enhancement of phosphorylation was observed at higher concentrations of the growth factors. The time course analysis of the tyrosyl phosphorylation of the proteins in normal cells in response to the PDGF stimulation showed that the phosphorylation patterns were roughly similar after 5, 10 or 15 min (which was the maximum) and declined thereafter. Therefore, in the shown experiments, the cells were stimulated with 30 ng/ml of PDGF for 15 min, and the attained phosphorylation levels were used as a reference in searching for constitutively activated molecules in the different transformed cells.

*c-Ha-*ras ^{Val12}*- and ODC-transformed cells show reduced protein tyrosine phosphorylation in response to PDGF stimulation*

We then compared both the basal and PDGF-mediated tyrosine phosphorylation levels of proteins in normal NIH3T3 fibroblasts to those in the NIH3T3 cells transformed by the c-Ha-*ras*^{Val12} oncogene and human ODC cDNA. To also assess the dose effects of the transforming proteins, in many experiments we examined two *ras*-transformed cell lines, the E2 and E4 cells (Hölttä *et al.*, 1988; Sistonen *et al.*, 1989b) and two ODC-transformed cell lines, the human ODC-overexpressing pLTR(S) cells (Auvinen *et al.*, 1992) designated here as Odc, and a cell line derived from the pLTR(S) cell-induced tumors in nude mice, designated as Odc-n, which express lower and higher levels of Ras and ODC, respectively. PDGF-AA stimulation of normal NIH3T3 cells resulted in a prominent increase in the tyrosine phosphorylation of proteins of about 190, 150, 56 and 52 kDa (data not shown), and stimulation with PDGF-BB in the tyrosine phosphorylation of proteins of about 190, 150, 85, 69, 56 and 52 kDa (Figure 1a). The cells transformed by c-Ha-*ras* (E4) and human ODC cDNA did not display constitutive phosphorylation of these proteins (except possibly for the 56 kDa protein) and showed only marginal increases in the tyrosine phosphorylation of the 190 kDa band and

other proteins upon PDGF stimulation (Figure 1a). Further kinetic analyses did not change this result. Of note, the tyrosine phosphorylation of the 190 kDa band comprising both the PDGF α- and β-receptors (Figure 1a) and that of the PDGF β-receptor (Figure 1b) in response to the PDGF-BB stimulation was largely abrogated in the Ras transformants. Also the ODC-transformed cells showed a clear, yet less extensive attenuation of the PDGF-BB-induced tyrosine phosphorylation of the 190 kDa protein band (Figure 1a) and PDGF β-receptor (Figure 1b). Similarly, there was a reduction in tyrosine phosphorylated PDGF α-receptor in the *ras*- and ODC-transformed cells (Figure 1c).

PDGF-BB-mediated tyrosine phosphorylation of PLCγ-1 and RasGAP is inhibited in ras- *and ODC-transformed cells*

In normal fibroblasts, PLCγ-1 is known to become activated following its association with and tyrosine phosphorylation by the PDGF receptor (Kim *et al.*, 1991). In spite of numerous studies (Hill *et al.*, 1990; Valius and Kazlauskas, 1993), the significance of PLCγ-1 activation for the cell physiology and mitogenic response remains elusive. Similarly, RasGAP is rapidly tyrosine phosphorylated upon stimulation with PDGF, but the tyrosine phosphorylation of GAP appears not be required for mitogenesis (Valius and Kazlauskas, 1993). Concerning *ras* transformation, conflicting results exist as to the tyrosine phosphorylation and activation of both PLCγ-1 and GAP by PDGF, which prompted us to clarify these issues.

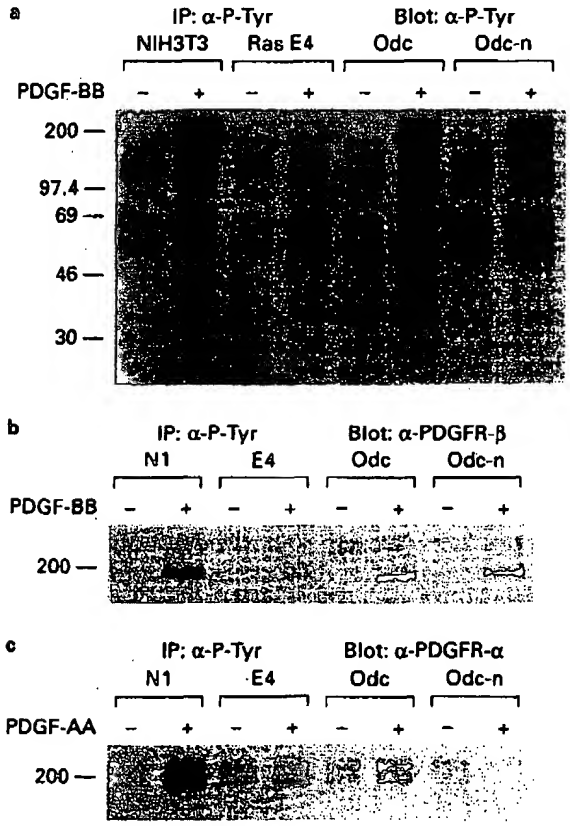

Figure 1 Analysis of tyrosine phosphorylated proteins in normal NIH3T3 fibroblasts (N1) and NIH3T3 cells transformed by c-Ha-ras^{Val12} oncogene (E4) and human ODC cDNA (Odc and Odc-n). The cells were stimulated (+lanes) or not (−lanes) with 30 ng/ml PDGF-BB (a and b) or PDGF-AA (c) for 15 min. SDS–PAGE (8%) resolved α-P-Tyr immunoprecipitated proteins were immunoblotted with monoclonal antibodies to phosphotyrosine (a), PDGF receptor-β (b) and PDGF receptor-α (c). The relative positions of molecular weight standards run in parallel are indicated on the left

The proteins from normal NIH3T3 cells and *ras*- and ODC-transformed NIH3T3 cells both stimulated or unstimulated with PDGF-BB were immunoprecipitated with 4G10 anti-phosphotyrosine antibodies, subjected to SDS–PAGE and blotted with specific antibodies to PLCγ-1 and GAP. When compared to the tyrosine phosphorylation level of PLCγ-1 in unstimulated and PDGF-stimulated normal cells, the *ras*- transformed E4 cells appeared to have a slightly elevated basal level of phosphorylated PLCγ-1, and showed only a marginal further increase upon PDGF stimulation (Figure 2a). The ODC-transformed cells, in turn, did not show a constitutive phosphorylation of PLCγ-1, but showed, like the Ras transformants, an attenuated response to PDGF, in particular the Odc-n cells (Figure 2a). The mixture of the monoclonal antibodies to PLCγ-1 used in the blotting is also known to recognize a 47 kDa adaptor protein Nck whose tyrosine phosphorylation is increased upon PDGF stimulation and whose overexpression can cause transformation of NIH3T3 cells (Chou *et al.*, 1992; Li *et al.*, 1992). Consistent with these reports, our immunoblotting of the anti-phosphotyrosine immunoprecipitates with PLCγ-1 revealed a small increase in the tyrosine phosphorylated 47 kDa protein in the PDGF-stimulated normal NIH3T3 cells. Interestingly, the cells transformed by *ras* and ODC appeared to show a small constitutive increase in the 47 kDa protein in the anti-phosphotyrosine immunoprecipitates (Figure 2b). A similar result was obtained by blotting with monoclonal antibodies to Nck (data not shown). However, its significance is difficult to say, as the changes in Nck did not always strictly correlate with the degree of morphological transformation. There were no differences in the total amounts of PLCγ-1 and Nck between the normal and *ras*- or ODC-transformed cells (Figure 2b).

Figure 2c illustrates the status of tyrosine phosphorylated RasGAP in the cells prior to and after PDGF-BB stimulation. In line with previous reports, normal NIH3T3 cells responded to PDGF with a marked increase in tyrosine phosphorylated GAP. The *ras*-transformed E4 cells appeared to have a slightly elevated basal level of tyrosine phosphorylated GAP and showed no response to PDGF-BB stimulation (Figure 2c). Likewise, in reciprocal experiments immunoblotting of the anti-GAP immunoprecipitates with monoclonal anti-phosphotyrosine antibodies revealed no enhancement of the tyrosine phosphorylation of GAP by PDGF in the *ras*-transformed cells. Unlike normal fibroblasts, the Ras transformants did not either show an increase in the tyrosine phosphorylated proteins co-immunoprecipitating with GAP after the PDGF treatment (data not shown). The ODC-transformed cells displayed also slightly elevated basal levels of tyrosine phosphorylated GAP, but differed from the *ras*-transformed cells by responding to PDGF-BB with a distinct increase in the tyrosine phosphorylation of GAP (Figure 2c).

Cells transformed by ras *and ODC show no constitutive tyrosine phosphorylation of the p85 subunit of PI3-K or activation of c-Src*

PI3-K which is composed of a regulatory 85 kDa subunit and catalytic 110 kDa subunit, catalyzes the formation of phosphatidylinositols phosphorylated at the D3 position. Several studies suggest an important role for PI3-K in the regulation of cell growth and malignant transformation (Cantley *et al.*, 1991; Parker and Waterfield, 1992). In our study, PDGF-BB stimulation of normal NIH3T3 cells resulted in a distinct increase in the p85 subunit of PI3-K in the anti-phosphotyrosine immunoprecipitates, while the *ras*- and ODC-transformed cells showed a smaller increase of p85 in the precipitates (Figure 2d). In reciprocal experiments, blotting of the anti-p85 immunoprecipitates with anti-phosphotyrosine antibodies revealed that p85 shows only a minor increase in tyrosine phosphorylation, but forms a complex with the tyrosine phosphorylated PDGF receptors. It is also notable that wortmannin, a potent inhibitor of PI3-K (Ui *et al.*, 1995), did not have any effect on the morphology of the *ras*- or ODC-transformed cells (up to 500 nM concentration) (Tuovinen and Hölttä, unpublished results).

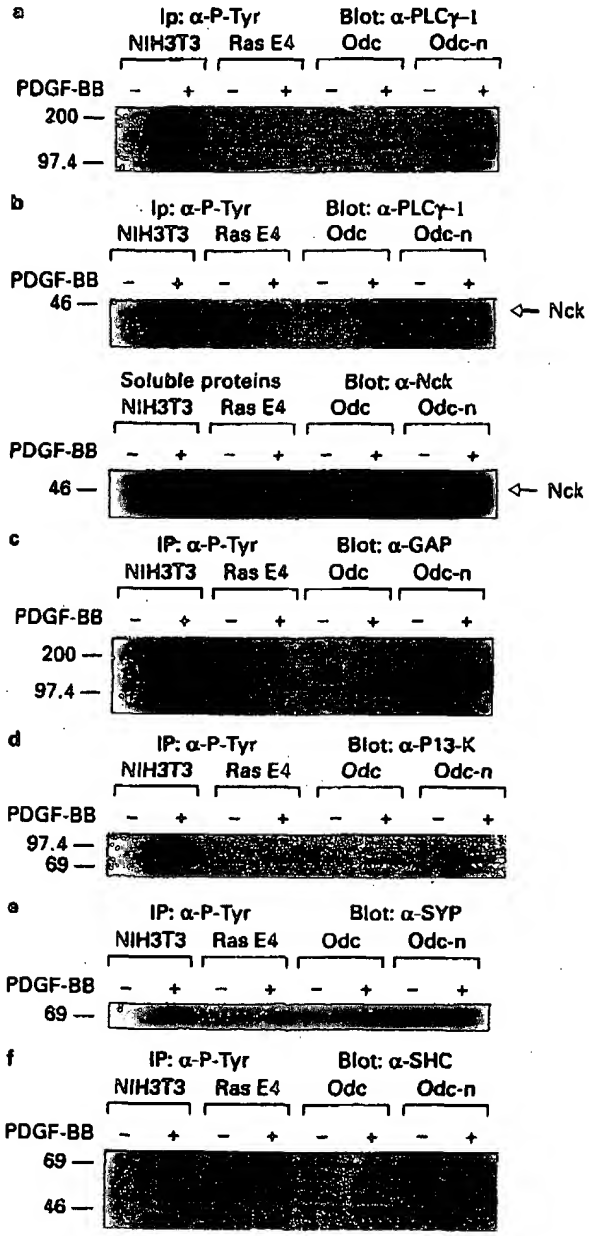

Figure 2 Phosphorylation status of various signaling molecules previously implicated in transformation in the normal and *ras*- or ODC-transformed NIH3T3 cells. The cells were untreated or treated with PDGF-BB and the total soluble proteins and α-P-Tyr immunoprecipitated proteins, resolved on SDS–PAGE (see Figure 1), were probed with specific antibodies to the indicated signaling molecules. The α-P-Tyr immunoprecipitates were blotted with antibodies to (a) PLCγ-1, (b) PLCγ-1 and Nck, (c) GAP, (d) PI3-K, (e) SYP and (f) SHC. Of note, the exposure time of the upper filter in (b) is three times longer than that in (a). The lower panel in (b) shows the total amount of Nck in soluble protein fractions which is the same in all the cell lines irrespective of the treatment. The same was true for the total amounts of the other signaling molecules. The arrows point to the Nck-proteins

Next, we studied the behaviour of c-Src in the PDGF-stimulated normal and transformed cells. Src family members are known to associate with the activated PDGF receptor (Kypta *et al.*, 1990), and play an important role in the cell's entry into S-phase and mitosis. Blotting analyses of c-Src in the anti-phosphotyrosine immunoprecipitates from cells stimulated with PDGF-BB or not did not show any dramatic changes in tyrosine phosphorylated Src either in normal, *ras*- or ODC-transformed cells. The *in vitro* kinase assays revealed a minor (about twofold) increase in the c-Src activity in normal cells upon stimulation with PDGF-BB, but the *ras*- and ODC-transformed cells were unresponsive and did not either show a constitutive elevation of the Src activity (Auvinen *et al.*, 1995, and data not shown).

Tyrosine phosphorylation of Syp and Shc is not involved in ras- *and ODC-transformation*

Protein tyrosine phosphatase Syp (PTP-1D/SHPTP2/SHP-2) associates directly with the PDGF receptors through its two SH2 domains and becomes highly tyrosine phosphorylated upon PDGF stimulation (Feng *et al.*, 1993; Vogel *et al.*, 1993). The major tyrosine phosphorylation site of Syp then serves as a docking site for the adaptor protein Grb-2 (Bennett *et al.*, 1994) which is associated with the guanine nucleotide exchange protein Sos-1 that, in turn, activates Ras (Buday and Downward, 1993). In agreement with earlier studies, we found that PDGF-BB stimulation of normal NIH3T3 cells triggered a marked increase in tyrosine phosphorylation of Syp. In contrast, stimulation of the *ras*-transformed E4 cells with PDGF-BB resulted in no or a very marginal increase in the tyrosine phosphorylated Syp, the basal level of which was low. Similarly, the ODC-transformed cells did not show constitutive tyrosine phosphorylation of Syp, but responded to PDGF, although to a lesser extent than the normal cells (Figure 2e).

Like Syp, the adaptor protein Shc can bind directly to PDGF β-receptor. Shc becomes tyrosine phosphorylated to a high degree upon ligand stimulation, and subsequently serves also as a docking molecule for Grb-2. Besides the possible function of Shc in mitogenesis, enforced expression of Shc has been found to result in morphological transformation of

NIH3T3 cells (Pelicci *et al.*, 1992). In normal NIH3T3 cells, PDGF-BB induced an increase in the tyrosine phosphorylation of all the three isoforms (47, 52 and 66 kDa) of Shc. The *ras*-transformed E4 cells did not display elevated basal levels of tyrosine phosphorylated Sch proteins, and showed no significant increase in the tyrosine phosphorylation of the 52 kDa Shc in response to PDGF-BB. The ODC-transformed cells did not show a constitutive elevation in tyrosine phosphorylation of Shc proteins either, and exhibited only moderate increases in the tyrosine phosphorylation of the 47 and 52 kDa isoforms in response to PDGF (Figure 2f). Similar results were obtained in converse experiments when blotting the anti-Shc immunoprecipitates with anti-phosphotyrosine antibodies. There were no significant differences in the total amounts of Shc proteins between the cell lines.

Sos-1 and Raf-1 show an electrophoretic mobility shift in the ras-, *ODC- and* v-src-*transformed cells*

Translocation of Sos-1 (complexed with Grb-2) from the cytoplasm into the activated receptor complex is evidently the key step in the activation of Ras at the plasma membrane, and no change in its intrinsic guanine nucleotide exchange activity may be required for signaling. Indeed, it has been documented that plasma membrane targeting of Sos-1 fully activates the Ras signaling pathway, and results in cell transformation (Aronheim *et al.*, 1994; Egan *et al.*, 1993). Following epidermal growth factor stimulation, Sos-1 is known to become phosphorylated on serine/threonine residues and its electrophoretic mobility is reduced (Burgering *et al.*, 1993; Rozakis-Adcock *et al.*, 1992, 1995). In our study, we found that stimulation of normal NIH3T3 cells with PDGF-BB also results in a decreased mobility of Sos-1 in SDS–PAGE. Interestingly, the *ras*-transformed cells displayed a constitutively retarded migration of Sos-1 (Figure 3a). This was also true for Odc-n cells expressing very high levels of ODC, while the transformants with lower ODC expression levels displayed only a slightly retarded mobility of Sos-1 and responded to PDGF with accentuation of the mobility shift (Figure 3a). Further, we found that the electrophoretic mobility of Sos-1 was also retarded in temperature sensitive (ts) RSVLA29 Rat-1 cells following v-*src* activation (Figure 3c).

Sos-1 converts inactive GDP-bound Ras to active GTP-bound Ras, which then interacts with and activates the serine/threonine kinase Raf-1 (Avruch *et al.*, 1994; Daum *et al.*, 1994). The requirement for Ras in Raf-1 activation is overcome by targeting Raf-1 to the plasma membrane (Leevers *et al.*, 1994; Stokoe *et al.*, 1994). The mechanism(s) by which the membrane bound Raf-1 becomes activated is not yet fully understood. The activated Raf-1 shows a reduced electrophoretic mobility (Daum *et al.*, 1994). In line with previous reports, we found that stimulation of normal NIH3T3 cells with PDGF-BB triggered an electrophoretic mobility shift in Raf-1. The *ras*-transformed cells in turn displayed a constitutively retarded migration of Raf-1 (Figure 3b). Surprisingly, the ODC-transformed cells also showed a reduction in the electrophoretic mobility of Raf-1, the extent of which depended on the expression level of ODC

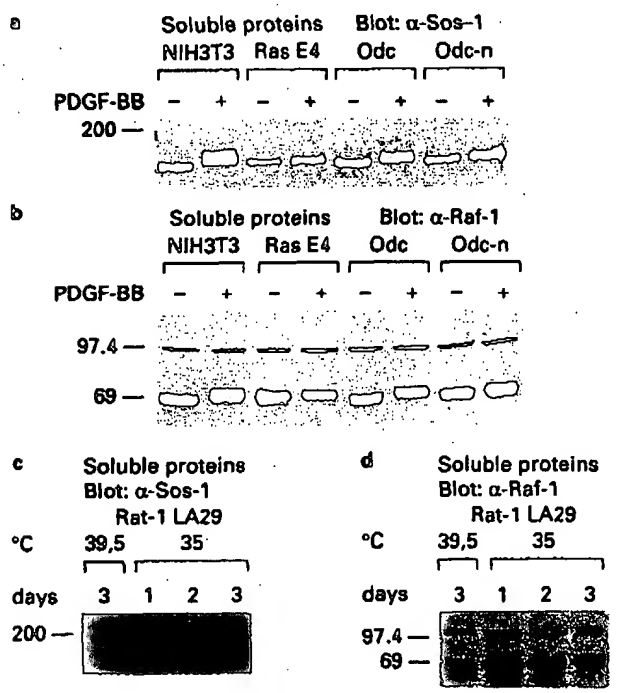

Figure 3 Sos-1 and Raf-1 show electrophoretic mobility shifts in the *ras*- and ODC-transformed cells, as well as in the Rat-1 LA29 cells upon v-*src*-induced transformation. Detergent-soluble total proteins were immunoblotted with antibodies to (a, c) Sos-1 (resolved in 6% SDS–PAGEs) and (b, d) Raf-1 (resolved in 8% SDS–PAGEs; note that the antibody also detects an additional 100 kDa Raf-related protein). The numbers above the lanes in panels (c) and (d) refer to days kept at the indicated temperatures

(Figure 3b). As reported for stably v-*src*-transformed cells (Reed *et al.*, 1991), the ts RSVLA29 Rat-1 cells were also found to exhibit a mobility shift of Raf-1 with similar kinetics to that of Sos-1 at the permissive temperature (Figure 3d).

Erk1 and Erk2 show no constitutive upregulation following ras-, *ODC- or* v-src-*transformation*

Activated Raf-1 initiates a phosphorylation cascade by phosphorylating first a MAP kinase (MAPK) kinase, also called MEK, which is a dual specificity kinase and phosphorylates MAPKs (Erks) on tyrosine and threonine residues, resulting in their activation. MAPKs are serine/threonine-specific kinases which play a key role in the activation of several transcription factors (Blenis, 1993; Cobb *et al.*, 1991; Crews and Erikson, 1993). According to recent evidence, constitutively active MAPK kinase can cause transformation of NIH3T3 cells (Cowley *et al.*, 1994; Mansour *et al.*, 1994), and MAPK has been found to be activated in cells transformed by several oncogenes, like *ras*, *src*, *raf* and *mos* (Gupta *et al.*, 1992; Leevers *et al.*, 1994; Mansour *et al.*, 1994; Samuels *et al.*, 1993). In agreement with earlier reports we found clear mobility shifts of Erk1 and Erk2 in NIH3T3 cells treated with PDGF-BB. Surprisingly, we did not detect a sustained tyrosine phosphorylation of Erk1 and Erk2 in cells stably transformed by *ras* or ODC (Figure 4a). We did not either detect any transient, intrinsic activation of Erk1 or Erk2 at different times of the cell cycle (data not shown). Neither did the ts RSVLA29 Rat-1 cells

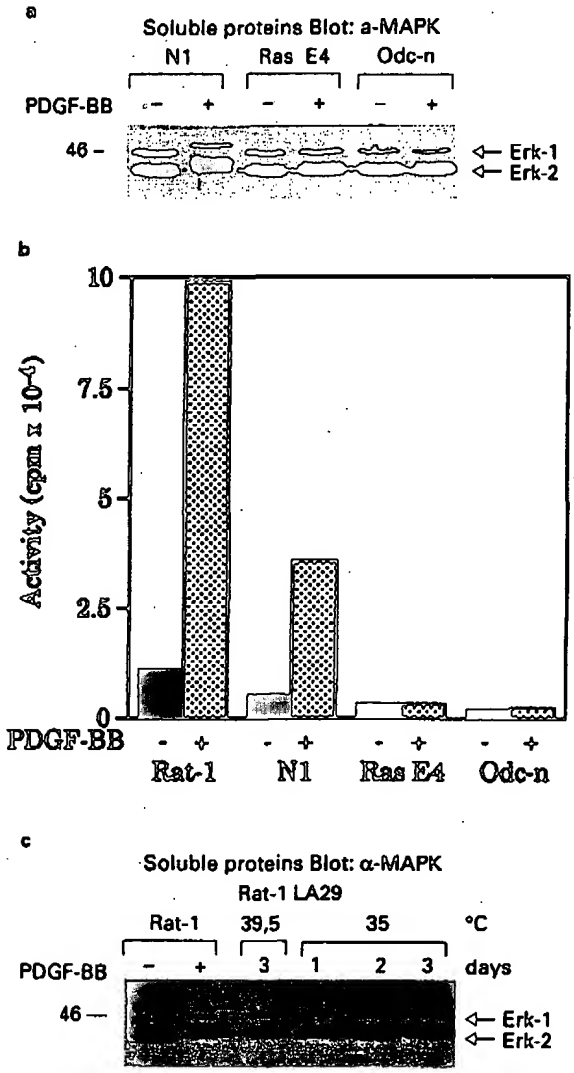

Figure 4 MAP kinases (Erk1 and 2) are not constitutively activated in *ras*-, ODC- or v-*src*-transformed cells. (a) Detergent-soluble proteins resolved in 12.5% SDS–PAGE immunoblotted with antibodies to MAPK show an electrophoretic mobility shift only in normal N1 cells upon PDGF stimulation. (b) The MAPK (Erk1 and Erk2) activities in normal and stably transformed cells measured as described in the Materials and methods. The black and dotted bars represent the non-stimulated or PDGF-BB-stimulated samples, respectively. (c) Detergent-soluble proteins from normal Rat-1 cells stimulated or not with PDGF-BB and from Rat-1 LA29 cells grown at restrictive (39.5°C) or permissive (35°C) temperature show an upshift of MAPKs (in 12.5% SDS–PAGE) only in the case of the normal cells upon PDGF stimulation

show any significant shifts in Erk1 or Erk2 mobilities upon v-*src* activation (Figure 4c). We then looked into the possibility that the activated Erk2 were translocated to the nucleus, but could not find any changes in the amount or mobility of Erk2 (or Erk1) in the nuclear fractions by immunoblotting (data not shown). In addition, we measured the MAPK (Erk1 and Erk2) activities of the normal or *ras*- and ODC-transformed cells by *in vitro* immunocomplex kinase assays with a synthetic peptide of myelic basic protein as a substrate (Figure 4b). The normal NIH3T3 cells responded to PDGF-BB with a 5–6-fold increase in MAPK activity and the respective stimulation of Rat-1 cells, used as a reference, yielded a tenfold MAPK activation (Figure 4b). The *in vitro* kinase assays did not reveal any increase in MAPK activity in the *ras*- or ODC-transformed cells (Figure 4b). It should also be noted that expression of the dominant-negative MAPK mutant effectively blocking Mos transformation (Okazaki and Sagata, 1995) only slightly reversed the morphology of the *ras*-transformed cells and had no effect on the phenotype of the ODC transformants. Moreover, we have found that treatment of the cells with a specific inhibitor of MEK (PD 098059) does not affect the transformed morphology or proliferation of the ODC-transformed cells, whereas the morphology of the Ras transformants is partially reversed towards the normal phenotype (Paasinen-Sohns and Hölttä, manuscript in preparation).

Tyrosine phosphorylation of STAT-1 (p91) or STAT-3 is not elevated in the Ras and ODC transformants

Recently, a novel pathway for growth factor activated transcription that involves interferon-responsive transcription factors termed STATs has been identified (Darnell *et al.*, 1994; Ihle, 1996). This pathway has been found to operate in the PDGF-mediated signaling in human fibroblasts (Silvennoinen *et al.*, 1993) and NIH3T3 cells (Wen *et al.*, 1995). We were therefore interested to compare alterations in this signaling pathway in the PDGF-stimulated normal NIH3T3 cells and Ras- or ODC-transformants. However, our blotting of the anti-phosphotyrosine immunoprecipitates from the normal or transformed cells did not reveal any significant increase in the tyrosine phosphorylated p91/84 STAT-1 (Figure 5a) or STAT-3 (data not shown) following PDGF treatment. Likewise, when p91 was immunoprecipitated from the N1 cells with specific antibodies, the proteins resolved by SDS–PAGE and blotted with monoclonal anti-phosphotyrosine antibodies, we did not detect any appreciable increase in the tyrosine phosphorylation of p91 in response to PDGF-BB stimulation (Figure 5b). No increases of p91 or p91 molecules with retarded mobility representing tyrosine and serine phosphorylated forms were either detected on blotting of the cytosolic or nuclear fractions from the PDGF-stimulated normal NIH3T3 cells, *ras*- or ODC-transformed cells with antibodies to p91 (Figure 5c). Neither did we detect PDGF-induced tyrosine phosphorylation of the JAK1 and JAK2 tyrosine kinases assumed to mediate p91 phosphorylation (Darnell *et al.*, 1994; Ihle, 1996) in these cells. Thus, we conclude that tyrosine phosphorylation of p91 does not play a substantial role in the PDGF-mediated activation of gene transcription in NIH3T3 cells under the physiological conditions. This notion is also supported by the recent finding on p91 knock-out mice showing no defect in responsiveness to any growth factor or cytokine except interferon (Durbin *et al.*, 1996). It is also clear from our studies that p91 is not constitutively activated in cells transformed by *ras* or ODC.

p70/85 S6 kinase is not involved in transformation

We have also studied the possible role played by the recently documented novel Ras-independent PDGF-signaling pathway leading to activation of p70/85 S6 kinase, Ming *et al.*, 1994) in transformation. Stimulation

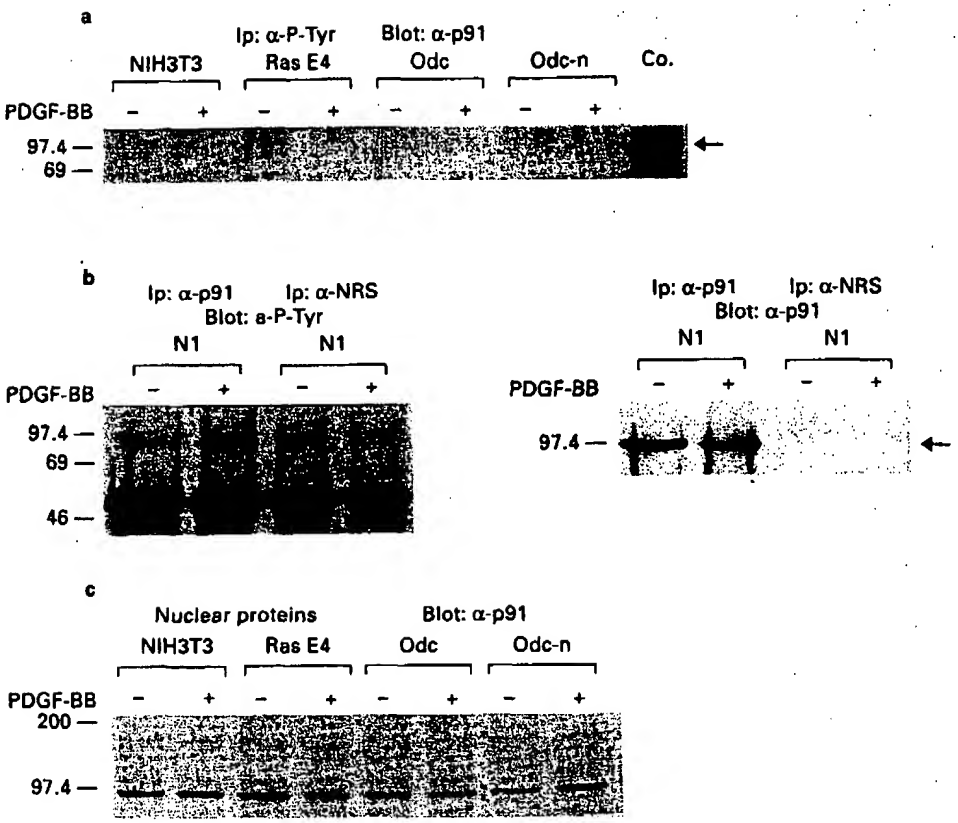

Figure 5 Tyrosine phosphorylation of p91 (STAT-1) is not elevated in the *ras*- and ODC-transformed cells. The immunoprecipitations with (**a**) α-P-Tyr antibodies or (**b**) α-p91 and α-NRS (normal rabbit serum) were resolved in SDS–PAGE and immunoblotted with antibodies to (**a**) p91 and (**b**, left) phosphotyrosine. The right part of the panel (**b**) shows the same filter as on the left, blotted with antibodies to p91. The faint signal seen in the left part of the panel (**b**) does not appear to be specific for p91. (**c**) Nuclear translocation of the p91 protein was neither seen in the PDGF-stimulated normal cells nor the *ras*- and ODC-transformed cells

of normal NIH3T3 fibroblasts with PDGF was found to result in an about threefold increase in the p70/85 S6 kinase activity. The *ras*-transformed NIH3T3 cells showed a similar basal S6 kinase activity to the normal cells, and responded with a slight (1.5-fold) increase in the kinase activity upon PDGF stimulation. The ODC-transformed cells displayed a slightly elevated basal S6 kinase activity, which was stimulated about twofold by PDGF (Paasinen-Sohns and Hölttä, unpublished data).

NIH3T3 cells transformed by ras *and ODC show repression of the PDGF α- and β-receptors*

It has become apparent that many oncogenes modulate the function of growth factor receptors and may induce autocrine loops (Baserga, 1994). In several previous studies (concerning PDGF β-receptors) the number of PDGF receptors has been found to be normal in *ras*-transformed NIH3T3 cells. However, we found that the *ras*-transformed E4 cells display a marked reduction in PDGF α- and β-receptors (Figure 6a and b). Blotting with the α-receptor antibodies showed that the levels of both the mature 190 kDa α-receptor and its 160 kDa precursor were decreased in the E4 cells (Figure 6a). The ODC-transformed cell lines, particularly the Odc-n cells appeared to show a still more significant reduction in the α-receptor levels (Figure 6a). Immunoblottings of the same cell extracts with the β-receptor antibodies in turn showed that the *ras*-transformed E4 cells display quite a significant decrease or total loss of the PDGF β-receptors (Figure 6b). The ODC-transformed cells, particularly the Odc-n cells, also displayed a clear decrease in the PDGF β-receptors (Figure 6b). Similar results were obtained with untreated cells and cells treated with PDGF-BB for 15 min, although an incipient downregulation of the PDGF receptors by PDGF (Heldin, 1992) was already apparent in some experiments at this time. To further verify these findings, we measured the receptor levels by [^{125}I]PDGF-AA and [^{125}I]PDGF-BB binding assays (Figure 6c). The binding of the radioactive ligands in the absence or presence of increasing (and saturating) concentrations of unlabeled PDGF-AA or PDGF-BB revealed that normal NIH3T3 cells have several-fold higher content of the PDGF β-receptors than the α-receptors (Figure 6c). Consistent with the immunoblotting results, both the *ras*- and ODC-transformed cells showed a marked decrease in the number of both PDGF α- and β-receptors.

In order to find out the mechanisms leading to the loss of the PDGF receptors in *ras*- and ODC-transformed cells, we examined the expression of the receptors at the mRNA level. Northern blot analysis revealed that both the *ras*- and ODC-transformed cells have markedly reduced levels of the PDGF α-receptor

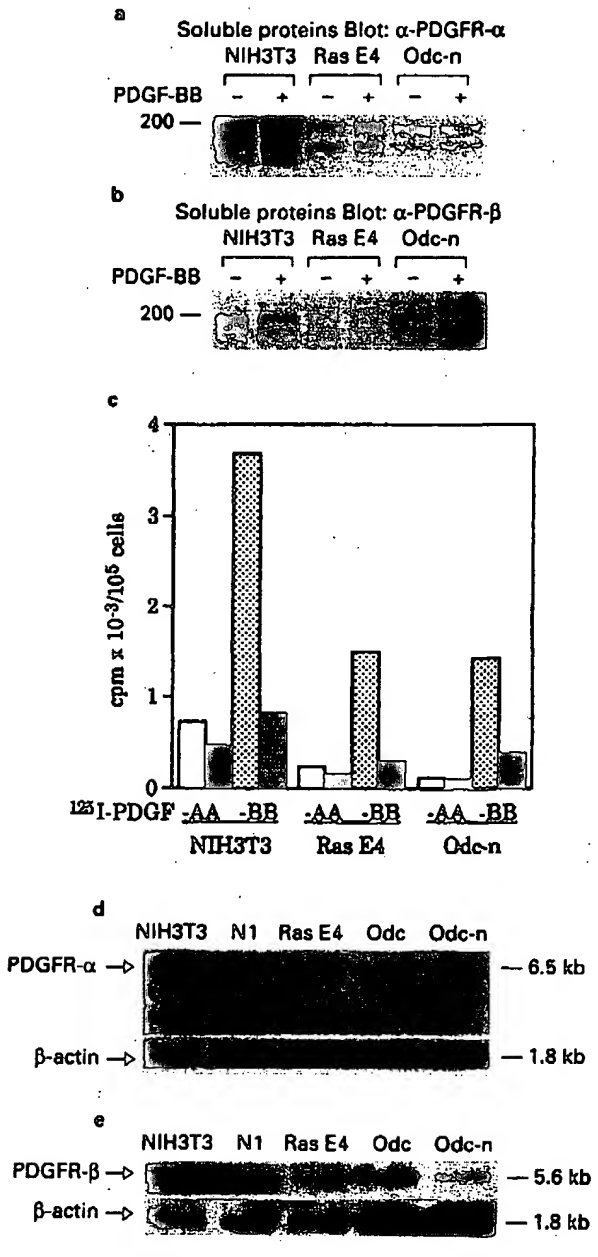

Figure 6 Downregulation of the PDGF α- and β-receptors in NIH3T3 cells transformed by *ras* and ODC. Detergent-soluble proteins resolved in SDS–PAGE were immunoblotted with antibodies to (a) PDGF α-receptor and (b) PDGF β-receptor. The receptor levels were also measured by [^{125}I]PDGF-AA and [^{125}I]PDGF-BB binding assays (c) as described in Materials and methods. One representative experiment out of three is presented. The white and dotted bars represent the binding of [^{125}I]PDGF-AA and -BB, respectively. The black and grey bars show the remaining binding of [^{125}I]PDGF-AA and -BB after saturation with non-radioactive PDGF-AA and -BB, respectively. The Northern blots probed with (d) PDGF α-receptor cDNA and (e) PDGF β-receptor cDNA show the expression of the PDGF receptor messenger RNAs in the *ras*- and ODC-transformed cells. The integrity and loading of RNA was controlled by hybridizing the blots with human β-actin cDNA probe. The sizes of the mRNAs are indicated on the right. The hybridization signals were quantified by phosphoimager (Molecular Dynamics, Inc.) scanning of the filters and adjusted to the β-actin mRNA levels. The relative values for the 6.5 kb PDGF-α mRNA (d) are NIH3T3 100, N1 141, RasE4 25, Odc 5 and Odc-n 3. The origin and the significance of the shorter PDGFR-α mRNA is not known currently. The relative values for the PDGF-β mRNA (e) are NIH3T3 100, N1 102, RasE4 12, Odc 26 and Odc-n 11

mRNA as compared with the normal NIH3T3 cells. The reduction of the α-receptor mRNA was most profound in the Odc-n cells (Figure 6d). Likewise, both the Ras E4 and Odc-n cells displayed a marked decrease in the levels of the PDGF β-receptor mRNA (Figure 6e). It is evident from these studies that the decreases in the receptor mRNAs are dependent on the expression levels of the transforming proteins, as shown for the ODC-transformed cell lines.

v-src- induced transformation of rat fibroblasts causes a preferential decrease in PDGF β-receptor levels

We also analysed the PDGF α- and β-receptor levels in the v-*src*-transformed rat fibroblasts (Rat-1 LA29) (Wyke and Stoker, 1987). Immunoblotting analyses showed that the number of the PDGF α-receptors remains unaffected or is only slightly reduced during the v-*src*-induced transformation in these cells (data not shown), while in v-*src*-transformed NIH3T3 cells the PDGF α-receptors appear to show a clear decrease (Lih *et al.*, 1996). But blotting of the Rat-1 LA29 cell lysates with antibodies to the β-receptor revealed a profound decrease in the amount of the PDGF β-receptors following v-*src* induction, correlating with the degree of morphological transformation (data not shown).

The oncogene expression levels determine the degree of aberration in signaling and may explain previous conflicting results

Since the observed downregulation of PDGF receptors in *ras*-transformed cells was in contrast with the previously published data, we looked into the possibility that the opposite results could be derived from different expression levels of the *ras* oncogene. Indeed, it was found that levels of the PDGF α- and β-receptors were decreased in an oncogene expression-dependent manner, the decrease being much smaller in the E2 cells expressing lower levels of *ras* than the E4 cells (Figure 7a and b). This was also reflected, as expected, in the PDGF-induced alterations in the signaling molecules and may explain the discrepancy in earlier studies (see Introduction). For example, the tyrosine phosphorylations of PLCγ-1 and RasGAP in response to PDGF stimulation were attenuated less in the E2 than E4 cells (Figure 7c and d).

The ras-, *ODC- and* v-src-*transformed cells display a constitutive increase in phosphorylation of c-Jun on serines 63 and 73*

The growth factor-induced signals amplified by the phosphorylation cascades culminate in changes in transcription factor activities, which often involves protein phosphorylation. Since the *ras*-, ODC- and v-*src*-transformed cells did not display activation of the MAPK or JAK-STAT pathways, but showed constitutive phosphorylation of Raf-1 and Sos-1, we examined the signaling into the nucleus and analysed the phosphorylation of the c-Jun and other transcription factors. Also, we examined the possible activation of the JNKs (c-Jun N-terminal kinases) (Dérijard *et al.*, 1994; Hibi *et al.*, 1993; Karin and Hunter, 1995), in these transformants. Previously, c-Ha-*ras* oncogene has been

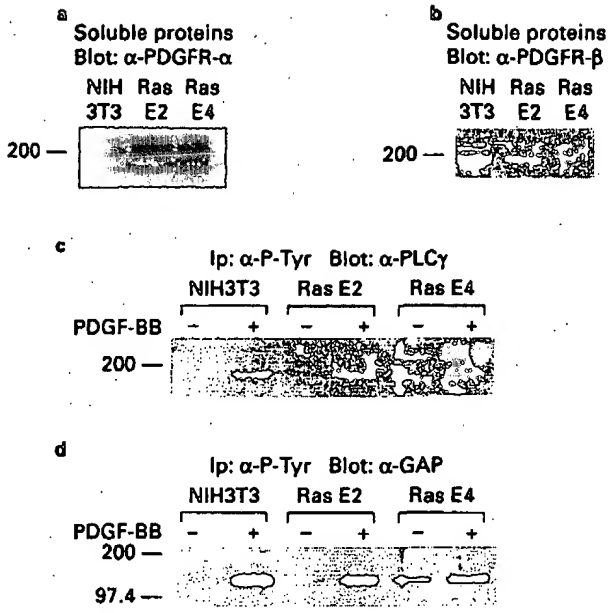

Figure 7 Downregulation of PDGF receptors and tyrosine phosphorylation of PLCγ-1 and GAP in an oncogene expression-dependent manner in *ras*-transformed cells. Detergent-soluble proteins resolved in SDS–PAGE were immunoblotted with antibodies to (a) PDGF α-receptor and (b) PDGF β-receptor. α-P-Tyr immunoprecipitated proteins resolved in SDS–PAGE were immunoblotted with antibodies to (c) PLCγ-1 and (d) GAP

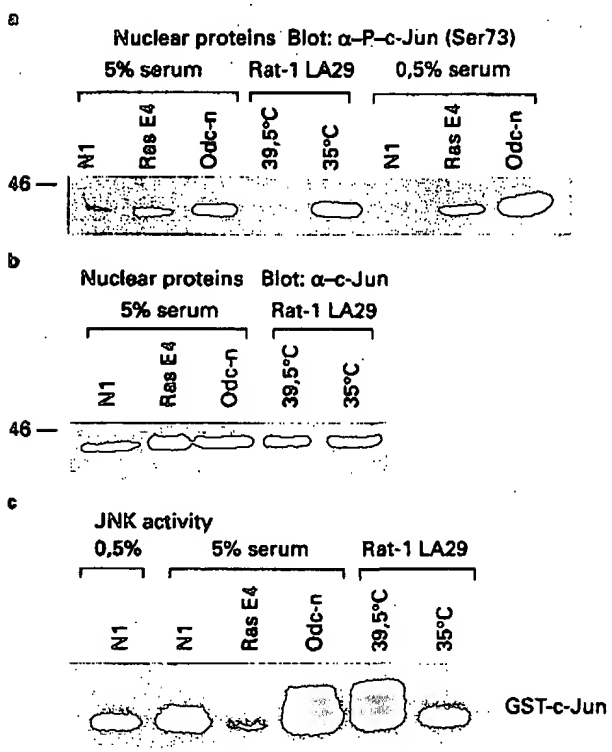

Figure 8 Phosphorylation of c-Jun on its aminoterminal activation domain (on serine 73) and JNK activity in cells transformed with *ras*, ODC and temperature-sensitive v-*src*. (a) Nuclear proteins from the normal and c-Ha-ras^{Val12}-oncogene or ODC-transformed cells grown in the presence of serum or starved for serum (24 h), and from ts v-*src*-oncogene expressing cells were resolved in SDS–PAGE and blotted with monoclonal antibodies specifically detecting c-Jun phosphorylated on serine 73. (b) The nuclear protein samples blotted with α-c-Jun antibodies show the total amount of c-Jun in the cells. (c) The phosphorylation of c-Jun(1–169)-GST substrate in JNK1 immunocomplex kinase assay. The unexpectedly high JNK activity in the Rat-1 LA29 cells at 39.5°C is likely due to a heat shock response. A similar result was obtained in the solid-phase JNK kinase assay

found to augment phosphorylation of c-Jun on its activation domain, as assessed by ^{32}P-orthophosphate labeling (Binétruy *et al.*, 1991; Karin and Hunter, 1995; Pulverer *et al.*, 1991). Our results obtained with the phospho-specific antibodies to c-Jun are in line with these observations. Whereas the normal N1 cells only exhibit a very faint phosphorylation of c-Jun on serine 73 when stimulated with serum (Figure 8a), the cells transformed by *ras* or ODC display a strong constitutive phosphorylation of c-Jun on serine 73 grown either in the presence or absence of serum growth factors. In addition, the induction of cell transformation by the ts v-*src* oncogene was found to result in serine 73 phosphorylation of c-Jun. It should be noted that these transformed cells showed also an increase in phosphorylation of c-Jun on serine 63 (data not shown), which is known to be the other, minor phosphorylation site in the transactivation domain (Hibi *et al.*, 1993). The expression level of c-Jun protein was only slightly elevated in the *ras*- and ODC-transformed cells relative to the normal N1 cells (Figure 8b), indicating that it is truly a question of increased phosphorylation of c-Jun in the transformants. Interestingly, our *in vitro* JNK assays (Figure 8c) revealed that JNK1 and JNK2 can not be solely responsible for the phosphorylation of c-Jun on its activation domain in these three transformed cell lines, indicating that there is still an unknown kinase capable of phosphorylating c-Jun on serines 63 and 73, important for transformation.

Discussion

Growth factor-induced signals may be transmitted from the cell surface to the nucleus through multiple signal transduction pathways. These signaling cascades are thought to play an important role in the control of cell proliferation, as the products of many oncogenes have proved to be mutationally activated forms of the normal signaling molecules. The currently held view is that there may be multiple routes leading to cell transformation, but the key unresolved question is whether these pathways finally converge to a common alteration in signaling dictatory for neoplastic transformation.

According to many previous results, cells transformed by *ras* become refractory to PDGF stimulation and this is not due to a loss of the receptors, but is caused by suppressed PDGF β-receptor autophosphorylation (Rake *et al.*, 1991; Tomáška and Resnick, 1993). However, we found a marked decrease in the β-receptor levels, and also of the α-receptors, in the *ras*-transformed cells. This was confirmed both by [^{125}I]PDGF-BB and [^{125}I]PDGF-AA receptor binding assays and immunoblotting analyses. Thus, both the ligand-unoccupied and total numbers of the receptors appear to be decreased. One explanation to the earlier discrepant results could be a lower Ras expression, as our studies revealed an oncogene expression-dependent decrease in the number of the PDGF receptors.

Recently, also Vaziri and Faller (1995) have found repression of the expression of PDGF-β receptor in *ras*-transformed 3T3 cells.

We further found that cells transformed by human ODC, which may be a mediator of the activation of many oncogenes (Auvinen *et al.*, 1992, 1995) through still unknown signaling mechanisms, display a marked decrease in the levels of both PDGF α- and β-receptors, in a manner similar to the *ras*-transformed cells. The ODC-transformed cells showed even a larger reduction in the PDGF α-receptors than the β-receptors. The v-*src*-transformed rat fibroblasts, in turn, showed a predominant reduction in the PDGF β-receptor levels. Besides the reduction in the PDGF receptors, we have found that the *ras*- and ODC-transformed cells also show decreased expression of the EGF receptor (Auvinen *et al.*, 1997 and data not shown), similar to that reported for the v-*src*-transformed cells (Wasilenko *et al.*, 1990). Further, insulin receptor expression has recently been reported to be reduced in *ras*-transformed cells (Baron-Delage *et al.*, 1994). In addition, we have previously found downregulation of the PDGF α- and β-receptors also in NIH3T3 cells transformed by the *neu* receptor tyrosine kinase (Lehtola *et al.*, 1991). Consequently, it appears that one reason, although not the only one, for the general unresponsiveness of different transformed cells to specific growth factors and serum (Greulich *et al.*, 1996; Samuels and McMahon, 1994; Yu *et al.*, 1993) may be a decrease or loss of the appropriate cell surface receptors. Some neoplastic cells, like the malignant glioblastomas and astrocytomas, can, however, express a specific receptor and its ligand, creating an autocrine loop (Heldin, 1992; Baserga, 1994). The production of the growth factors may also lead to receptor downregulation. However, this is not the mechanism for the downregulation of the PDGF receptors in our *ras*- or ODC-transformed cells, as we have not detected any increased expression of PDGFs by them. This conclusion is also supported by our finding of no constitutive activation of the upstream signaling molecules in these cells. Therefore, we postulate that the decrease in the receptor levels in these and probably many other transformed cells rather reflects negative feed-back regulation of their expression by the constitutively signaling oncoproteins.

A vast number of signaling molecules have been implicated in cell transformation, but it has remained unresolved whether any of them could represent a common element in the transformation process. The *ras*-transformed cells exhibited a slightly elevated basal tyrosine phosphorylation of GAP, which was not increased by PDGF treatment. This lack of GAP phosphorylation might well account for the profound decrease in the β-receptors, and the known inability of the residual α-receptors to associate with GAP (Bazenet and Kazlauskas, 1993). Unlike *ras*, the ODC-transformed cells did not show any constitutive increase in GAP phosphorylation. It also appears from our data that the tyrosine phosphorylation of GAP is dependent on the expression level of oncoproteins, which may explain the previously reported controversial results in the case of *ras* transformation. The same may hold for the conflicting results concerning the activation and tyrosine phosphorylation of PLCγ-1 by PDGF in the *ras*-transformed cells (see Introduction).

The adaptor proteins Syp and Shc, which were found to associate both with the PDGF α- and β-receptors in normal cells (Paasinen-Sohns and Hölttä, unpublished data), were found to show no elevation of basal phosphorylation and showed only marginal, if any, increases in tyrosine phosphorylation in response to PDGF treatment in the *ras*-transformed cells, while the ODC-transformed cells showed a detectable response. In addition, there were clear differences in tyrosine phosphorylation of the different signaling molecules in the PDGF-stimulated *ras*- and ODC-transformed cells. This differential behaviour might be related to the fact that the ODC transformants displayed a smaller decrease in the β- than α-receptors, while the opposite was true for the Ras-transformants. It should, however, also be taken into account that the proteins may undergo differential dephosphorylations by phosphotyrosine phosphatases, such as Syp, or there could still exist a third PDGF receptor (γ-receptor).

Interestingly, the *ras*-, v-*src*- and ODC-transformed cells showed a shift in the electrophoretic mobility of Sos-1, similar to that seen in the PDGF-stimulated normal 3T3 cells. This mobility shift of Sos-1 is known to be due to its phosphorylation on serine/threonine residues. Recent evidence shows that the phosphorylation of Sos-1 is associated with its dissociation from the cell surface receptor complexes or Grb2 and potential feed-back inactivation (Rozakis-Adcock *et al.*, 1995), which notion is also supported by our findings (unpublished data). The C-terminus of Sos-1 has several potential phosphorylation sites for MAPK, and Sos-1 has been documented to become phosphorylated by MAPK both *in vitro* and *in vivo* (Cherniack *et al.*, 1994; Rozakis-Adcock *et al.*, 1995). However, in our study we did not find the activity of MAPK (Erk1 and Erk2) to correlate with the phosphorylation state of Sos-1 in the *ras*- and ODC-transformed NIH3T3 cells and v-*src* transformed rat fibroblasts, suggesting that the phosphorylation of Sos-1 is performed by another kinase. Interestingly, Raf-1 showed a mobility shift similar to that in Sos-1 in the normal and *ras*- and ODC-transformed cells untreated or treated with PDGF, and in v-*src*-transformed rat cells. It remains to be seen whether it is a question of the same, possibly a novel kinase that phosphorylates both Sos-1 and Raf-1. As we did not find a correlation between the activity of JNKs and phosphorylation of Sos-1 and Raf-1 either, JNKs may be discounted. The electrophoretic mobility shift of Raf-1 resulting from its phosphorylation on serine residues is known to be associated with its activation (Daum *et al.*, 1994), but the shift is not always a good indicator of Raf-1 activation (Samuels *et al.*, 1993) and may also reflect feed-back inhibition. Raf-1 has previously been found to become constitutively activated and show a mobility shift in v-*src*-transformed cells (Reed *et al.*, 1991), and c-Ha-*ras*-transformed cells grown in the presence of serum (Morrison *et al.*, 1988), but not in the serum-starved Ras-transformants, which instead displayed overexpression of Raf-1 (Reed *et al.*, 1991). The fact that we observed a mobility shift in Raf-1 also in the c-Ha-*ras*-transformed NIH3T3 cells grown in low serum might be explained by higher expression levels of the Ras oncoprotein, since at least in the ODC-transformed cells the mobility shift of Raf-1 was dependent on the expression level of ODC. However, while in *ras*-

transformed cells this shift was associated with an increase in the activity of Raf-1 (in the immunocomplex assay), the ODC-transformed cells did not show any activation of Raf-1. Therefore we conclude that, at least in the case of ODC transformation, the shift in the electrophoretic mobility of Raf-1 represents negative feed-back regulation.

A number of studies have implicated MAPK to have an essential role in the receptor tyrosine kinase-mediated signal transduction and cell proliferation. Further, inhibition of MAPK by the MAPK phosphatase MKP-1 has been shown to block the induction of DNA synthesis in fibroblasts by an activated Ras (Sun *et al.*, 1994). The activity of MAPK has also been reported to be constitutively elevated in NIH3T3 cells transformed by *ras* and v-*src* (Gupta *et al.*, 1992; Leevers *et al.*, 1994). However, we did not find any constitutive elevation of MAPK (Erk1 and Erk2) in the *ras*- or ODC-transformed NIH3T3 cells, while a clear increase in MAPK (Erk1 and Erk2) activity was seen upon PDGF stimulation of the normal NIH3T3 cells. Neither did we detect any significant electrophoretic mobility shifts in Erk1 or Erk2 following v-*src*-induced transformation of rat fibroblasts, although such shifts were clearly detected in the control Rat-1 cells in response to PDGF. One explanation to the discrepant results could be that some of them are derived from transient transfection experiments and others, like ours, are based on stably transformed cells. Based on these findings, we conclude that the MAPK (Erk1 and ERk2) pathway is not responsible for the propagation of the proliferative signals and maintenance of the transformation by the ODC-, *ras*- and v-*src*-oncogene. This notion is further supported by our studies on the effect of dominant negative MAPK mutant in these cells. There are also a few other studies indicating that the activation of MAPK does not correlate with transformation (Alessandrini *et al.*, 1996; Gallego *et al.*, 1992; Greulich *et al.*, 1996; Samuels *et al.*, 1993). In the case of *ras*, however, we cannot totally exclude the possibility that the low basal activity of MAPK would contribute to transformation. Our data also show that the JAK-STAT-1 (or STAT-3) signaling pathway is not constitutively activated in the *ras*- or ODC-transformed NIH3T3 cells, suggesting that it is not critically involved in the cellular transformation process. Besides, this pathway was found to contribute very little, if at all, to the PDGF-mediated responses in normal NIH3T3 fibroblasts. It remains, however, to be determined whether the other recently discovered members of JAKs and STATs (Darnell *et al.*, 1994; Ihle, 1996) could play a role in transformation. Our data likewise exclude the possibility that the Ras-independent pathway leading to p70/85 S6 activation is involved in transformation.

But, consistent with our original hypothesis, we found at least one common alteration in signaling, the constitutive phosphorylation of c-Jun on its transactivation domain, which can easily be envisioned to play an important role in transformation. It is worth noting, that thus far, the analyses of the phosphorylation of c-Jun have been only based on [^{32}P]orthophosphate labeling studies, which may be subject to errors due to the changes in the [^{32}P] uptake, and specific radioactivity of the phosphate pools. The availability of phospho-specific antibodies now circumvents these problems, and may give a more reliable picture of the phosphorylation status of the proteins. Previous studies (Binétruy *et al.*, 1991; Pulverer *et al.*, 1991) although not all of them (Baker *et al.*, 1992) have shown that the phosphorylation of c-Jun (on serines 63 and 73) is associated with its increased transcriptional capability, possibly via binding to the CBP co-activator (Karin and Hunter, 1995) or other transcription factors. In any case, this domain is known to be essential for the transforming potential of c-Jun (Hartl and Vogt, 1992), suggesting that one clue to transformation could be in transcription of some specific target genes.

What is then the kinase(s) responsible for the phosphorylation of c-Jun in these cells? The JNK1 and JNK2 that are distantly related to MAPK, are thought to be the principal kinases phosphorylating c-Jun on serines 63 and 73. In cells transiently transfected with the *ras*- or v-*src*-oncogenes, the activity of JNKs has been found to be moderately elevated (Dérijard *et al.*, 1994; Minden *et al.*, 1995), and our studies show that in ODC-transformed cells the activity of JNK1 is highly elevated, which would give a reason to speculate that the answer is in the activation of JNK. However, we did not find a correlation between the activities of JNKs and the phosphorylation of c-Jun (on serine 73) in the *ras*- and v-*src*-transformed cells, suggesting that there is still an uknown, major kinase capable of phosphorylating the activation domain of c-Jun. It has recently been reported that c-Raf/Mil could phosphorylate c-Jun on serines 63 and 73 (Radziwill *et al.*, 1995), but we did not detect increased phosphorylation of c-Jun(1–169)-GST fusion protein in the anti-c-Raf immunoprecipitates from the ODC- or *ras*-transformed cells (Paasinen-Sohns and Hölttä, manuscript in preparation). One potential candidate could be the proline-directed protein kinase phosphorylating c-Fos, termed FRK (Deng and Karin, 1994), as the sequences surrounding the phosphorylation sites in c-Jun and c-Fos are highly homologous. It is also interesting to note that, the recently proposed hypothesis that the activation of JNK and concurrent inhibition of Erk would be a decisive factor for apoptosis (Xia *et al.*, 1995), does not hold for the ODC-transformed cells which display such a situation.

In conclusion, our results show that the ODC-, c-Ha-*ras*- and v-*src*-transformed cells all display a reduction or loss of specific growth factor receptors, which is evidently caused by a negative feed-back regulation at the level of mRNA expression. Significantly, although a great number of the intermediates of signaling, such as Syp, Shc, Src, Nck, PI3-K, Sos-1, Ras, Raf-1 and MAPK kinase, have previously been shown to have transforming activity or to be elevated in different cancer cells, it is apparent from our studies that very few, if any, of them can be considered to be characteristic of transformed cells in general. Only Sos-1 and Raf-1 exhibited a constitutively retarded electrophoretic mobility in all these three transformants. We assume that these changes reflect negative feed-back phosphorylation loops (that probably exist also in the normal ligand-activated cells), because Ras is known to act downstream of Sos-1 and ODC acts even downstream of the nuclear transcription factors,

such as c-Myc (Bello-Fernandez *et al.*, 1993; Hölttä *et al.*, 1994), and no increase in Raf-1 activity was observed in the ODC transformants. Interestingly, Erk1 and Erk2 seem not to be responsible for the mobility shifts of Sos-1 and Raf-1 or the driving force for the proliferation and maintenance of the transformed phenotype of the ODC-, *ras*- or v-*src*-transformed cells. The JNK, JAK-STAT-1 (or STAT-3) and S6 kinase signaling molecules are not the common denominators in transformation, either. But the observed constitutive tyrosine phosphorylation of some proteins (Auvinen *et al.*, 1995) and persistent serine/threonine phosphorylation of Raf-1, Sos-1 and c-Jun in the c-Ha-*ras*-, v-*src*- and ODC-transformed cells suggests that there is a tyrosine kinase and a serine/threonine kinase, or a dual-specificity kinase, constitutively active in these cells. Moreover, we have recently found that the morphological transformation by ODC, c-Ha-*ras*^{Val12}- and v-*src*- oncogene are reversed by selective inhibitors of protein tyrosine kinases (Auvinen *et al.*, 1995) and serine/threonine kinases (Paasinen-Sohns and Hölttä, unpublished data). Identification of these kinases and full understanding of the meaning of the constitutive phosphorylation of c-Jun activation domain will be important. The answers to significant questions, such as how does the phosphorylation of c-Jun affect its interaction with other proteins, are other transcription factors possibly phosphorylated, and which target genes are specifically activated, could give important clues to the ultimate molecular mechanism of cancer development.

Materials and methods

Cell culture

The cell lines used were: NIH3T3 cells and their stable transfectants expressing the neomycin resistance gene alone (N1), c-Ha-*ras* oncogene (pGEJ6.6) at different levels (E2 and E4) (Hölttä *et al.*, 1988; Sistonen *et al.*, 1989b) and human ornithine decarboxylase cDNA (Odc) (Auvinen *et al.*, 1992). Odc-n cells were derived from the Odc cell-induced tumors in nude mice (Auvinen *et al.*, 1997). The Rat-1 fibroblasts, and temperature sensitive (ts) RSVLA29 Rat-1 cells carrying the ts339*src* gene of RSV-B77 have been described earlier (Wyke and Stoker, 1987).

The NIH3T3 and Rat-1 cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with antibiotics and 5% fetal calf serum (Bioclear). The ts v-*src* cell lines were cultured in RPMI-1640 medium containing the antibiotics and 5% fetal calf serum.

Antibodies

Polyclonal antibodies to the PDGFR-α and -β (R7 and R3) were kind gifts from C-H Heldin and L Claesson-Welsh. Polyclonal p91c was provided generously by JE Darnell Jr. Monoclonal agarose-conjugated anti-phosphotyrosine 4G10, monoclonal antibodies to Ash/Grb-2, Nck and PLCγ-1, polyclonal antibodies to GAP, SHC, Sos-1, SHPTP2/SYP and C-terminal part of the Erk1 MAP kinase R2, and whole antisera to JAK1, JAK2 and the p85 subunit of PI3-K were purchased from Upstate Biotechnology Inc. Monoclonal anti-phosphotyrosine antibodies (clone PT-66) were from Sigma. Monoclonal antibodies to MAP kinase recognizing Erk1 and Erk2, were purchased from ZYMED Laboratories. Anti-v-src-antibody 327 was from Oncogene Science. Polyclonal antibodes raised against Raf-1 (C-12), c-Jun/AP-1 (N) and JNK1 (C17) were purchased from Santa Cruz Biotechnology. Monoclonal phospho-specific c-Jun (Ser 73) and c-Jun (Ser63) antibodies were from New England BioLabs.

Cell lysates and immunoprecipitations

NIH3T3 cells, their derivatives and Rat-1 cells were grown for 2 days and starved for 20–24 h in medium containing 0.5% fetal calf serum. The cells were stimulated with 30 ng/ml of human recombinant PDGF-AA or -BB (Upstate Biotechnology Inc.) for 15 min at 37°C. In transformation assays the temperature-sensitive v-*src* cells were kept at the restrictive temperature (39.5°C) for 2 days and then shifted to the permissive temperature (35°C).

Cells were lysed in non-ionic detergent buffer (50 mM HEPES pH 7.0, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA 100 mM NaF, 10 mM NaPPi, 1 mM AEBSF, 2 mM sodium orthovanadate, 10 μg/ml aprotinin/leupeptin) and nuclei were removed by centrifugation. Protein concentrations of the soluble proteins were then determined by BioRad kit.

40 μg of the detergent-soluble proteins were suspended in Laemmli's sample buffer and boiled for 5 min. In immunoprecipitation analyses, equal amounts of proteins (500–1500 μg) were immunoprecipitated with specific antibodies or nonimmune sera for 1 h at 4°C. Complexes of mAbs were harvested using agarose-beads with covalently conjugated goat anti-mouse IgG and pAbs respectively with agarose-beads covalently conjugated with goat anti-rabbit IgG (Sigma Chemical Co.). Anti-phosphotyrosine immunoprecipitations were carried out with agarose-conjugated monoclonal anti-phosphotyrosine 4G10. The resulting immunocomplexes were washed five times with lysis buffer and the specificity of the phosphotyrosine antibodes was controlled by addition of 50 mM phenylphosphate for competition with the samples. Samples suspended in Laemmli's buffer were then boiled for 5 min. All the analyses were repeated at least three times.

Isolation of nuclear fractions

Cells were suspended in hypotonic lysis buffer (10 mM Tris pH 7.5, 10 mM NaCl, 3 mM $MgCl_2$, 25 mM β-glycerophosphate, 2 mM sodium orthovanadate, 10 μg/ml aprotinin/leupeptin, 1 mM PMSF, 50 μM NaF, 2 mM DTT, 0.5% NP-40), and the nuclei were collected by centrifugation at maximal speed in an Eppendorf centrifuge for 20 s. The pellet was resuspended in the hypotonic lysis buffer, and thereafter sonicated on ice for 10 s. The nuclear lysates were cleared by centrifugation. The protein concentrations were measured and the samples were prepared for SDS–PAGE as described above.

Western blotting

The proteins were resolved by 8% SDS–PAGE (unless stated otherwise) and transferred onto nitrocellulose (BioRad Trans-Blot Transfer Medium). The filters were incubated in blocking buffer (25 mM Tris pH 8.0, 125 mM NaCl, 0.1% Tween, 2% BSA, 0.1% NaN_3) overnight, and then with specific antibodies for 4–6 h. The filters were rinsed five times in the washing buffer (10 mM Tris pH 8.0, 150 mM NaCl, 0.05% NP-40, 0.05% Tween). For the detection by ECL Western Blotting system (Amersham RPN 2106, Boehringer Mannheim), mAbs and pAbs were probed with peroxidase-conjugated rabbit IgGs to mouse IgGs and swine IgGs to rabbit IgGs (DAKO-immunoglobulins), respectively. The filters were washed five times with the washing buffer, 15 min in high salt buffer (10 mM Tris pH 8.0, 300 mM NaCl), and finally three times in TBS (10 mM Tris pH 8.0, 150 mM NaCl) before the incubation with ECL detection reagents. The filters were exposed to FUJI RX film.

Receptor binding assay

Cells were grown in 12-well plates for 2 days, washed once with cold binding buffer (0.9 mM $CaCl_2 \cdot 2H_2O$, 0.49 mM $MgCl_2 \cdot 6H_2O$, 137 mM NaCl, 2.7 mM HCl, 1.42 mM KH_2PO_4, 8.4 mM $Na_2HPO_4 \cdot 2H_2O$, 0.1% BSA) and PDGF-AA or -BB (Upstate Biotechnology Inc.) was added to the controls (at different concentrations or in 100-fold molar excess to the radioactive ligand) in 0.5 ml binding buffer for 10 min. After saturation with unlabeled PDGF, 55 000 c.p.m. of ^{125}I-labeled PDGF-AA (kindly provided by C-H Heldin) or PDGF-BB (Amersham IM213) was added to the wells and the plates were incubated at 4°C for 50 min. The wells were washed five times with 1 ml binding buffer and lysed finally in 0.5 ml lysis buffer (1% Triton X-100, 10% glycerol, 20 mM HEPES pH 7.4) for 30 min at room temperature and counted in a γ-counter. The parallel cultures were trypsinized and the number of cells was counted.

Assay of Src kinase activity

Cells were lysed and immunoprecipitated with monoclonal α-v-Src antibody. The immunoprecipitates were washed five times with the lysis buffer, once with the lysis buffer containing 0.3 M NaCl and finally twice with kinase buffer (20 mM Tris pH 7.5, 5 mM $MgCl_2$, 0.1 mM EGTA, 0.1% TX-100, 0.5 mM sodium orthovanadate). The tyrosine kinase activity of $pp60^{Src}$ was then assayed towards the synthetic peptide KRLIEDNEYAARQG as described earlier (Auvinen *et al.*, 1995).

Assay of MAPK activity

Cells were lysed and immunoprecipitated with polyclonal α-rat MAPK R2 as described above. The washings were completed with kinase assay buffer (25 mM HEPES pH 7.4, 10 mM $MgCl_2$, 0.5 mM EGTA, 25 mM β-glycerophosphate, 50 μM NaF, 2 mM DTT, 0.5 mM sodium orthovanadate). The reaction was started by adding a mix in 25 μl volume containing 50 μM cold ATP, 5 μCi/nmol [γ-^{32}P]ATP (3000 Ci/mmol) (Amersham) and 25 μg MAP kinase substrate peptide (APRTPGGRR) (Upstate Biotechnology Inc.) in assay buffer to the immunoprecipitates. The samples were incubated at 30°C for 15 min, whereafter the reactions were terminated by addition of 5 μl 100 mM ATP to each tube on ice. The incorportion of [^{32}P] into the peptide was measured by counting the radioactivity retained on an ion-exchange paper (P81, Whatman) after washing off the free ATP by 0.5% phosphoric acid.

Assay of JNK activity

For JNK immunocomplex assay the cells were lysed as above and immunoprecipitated with polyclonal α-JNK1 (C-17) antibody, which also cross-reacts with JNK2 to a lesser degree. The kinase reaction was performed as described in the MAPK assay using 2 μg of human c-Jun (1–169)-GST (Upstate Biotechnology Inc.) as a substrate. After 15 min at 30°C the reaction was stopped by adding Laemmli's sample buffer to the supernatant and the samples were resolved by SDS–PAGE and autoradiographed. The solid-phase JNK assay was performed as described (Hibi *et al.*, 1993) using agarose conjugated c-Jun (1–169)-GST (Upstate Biotechnology Inc.) for capturing the JNKs and as a substrate.

Northern blotting

Polyadenylated RNA was isolated by oligo(dT) chromatography from cell lysates (Sistonen *et al.*, 1989b). 8 μg aliquots of RNA were size-fractionated on 0.8% agarose gels containing 2.3 M formaldehyde and transferred to Hybond-N nylon filters (Amersham) in 6 × SSC. RNA was immobilized by Stratagene crosslinker. Prehybridizations were performed in 50% deionized formamide, 5 × Denhardt's solution (0.1% BSA, 0.1% Ficoll, 0.1% polyvinylpyrrolidone), 0.5% SDS, 6 × SSPE, 0.2 mg sonicated salmon sperm DNA/ml at 42°C overnight. Hybridizations were performed under the same conditions with ^{32}P-dCTP-labeled 4.5 kb PDGFR-α cDNA (obtained from J Escobedo) or 3.2 kb PDGFR-β cDNA (from American type culture collection, clone RP41) probes generated using a multiprime DNA labeling kit RPN.1601Z (Amersham). The membranes were washed 2 × 15 min at room temperature and 3 × 10 min at 60°C in 1 × SSC, 0.1% SDS before autoradiography. The integrity and loading of RNA was controlled by hybridizing the blots with a human β-actin cDNA probe.

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